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Protein delivery from polymeric matrices

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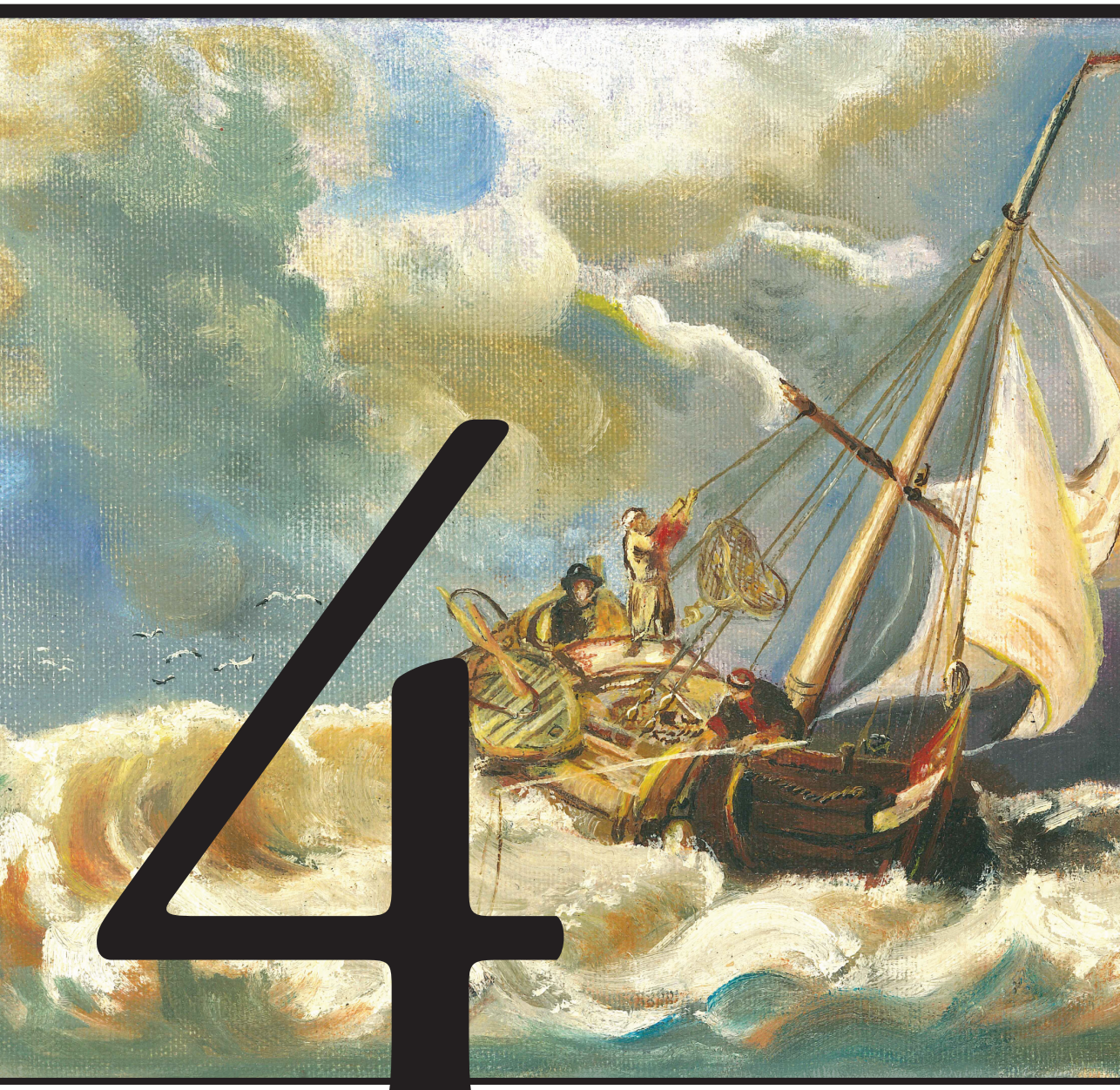

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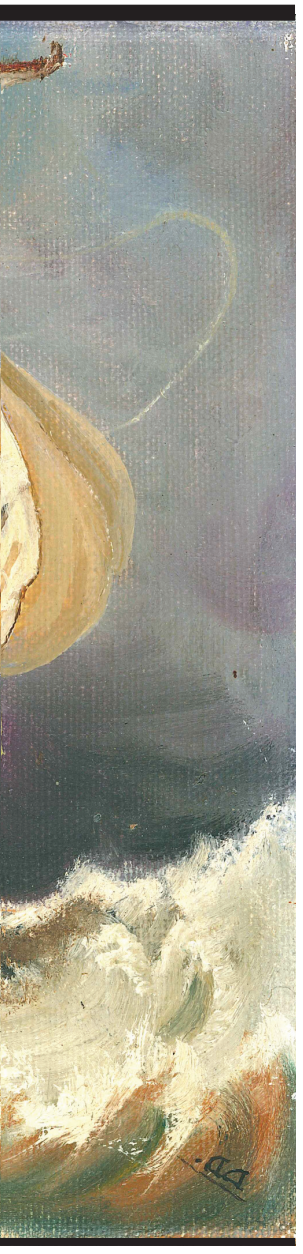

PROTEIN STABILITY DURING HOT MELT EXTRUSION: THE EFFECT OF EXTRUSION TEMPERATURE, SUGAR GLASS PRE-STABILIZATION AND HYDROPHILICITY OF POLYMERS

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ABSTRACT

Hot melt extrusion (HME) is a simple and continuous process to produce polymer implants for the controlled delivery of proteins. However, during this process the protein is exposed to harsh conditions, which could impair its integrity.

In this study, the effects of extrusion temperature, sugar glass pre-stabilization and hydrophilicity of polymers on stability of two model proteins after HME were investigated. Our results show that low extrusion temperature and sugar glass pre-stabilization with inulin, in particular at an intermediate extrusion temperature, improved the stability of the model proteins alkaline phosphatase and β-galactosidase. Furthermore, the thermal stability of the proteins was found to obey Arrhenius' Law and was predictive for trends in stability during HME. However, the use of hydrophilic polymers had an ambiguous effect on the stability of proteins after HME.

Taken together, our findings clearly show the potential of HME for the development of controlled release polymeric formulations containing therapeutic proteins. In particular, low extrusion temperatures and pre-stabilization with inulin improve the stability of proteins during HME.

Due to confidentiality reasons, the name of one of the polymers used in this Chapter can not be disclosed and is replaced by 'PEX'.

INTRODUCTION

The delivery of therapeutic proteins requires a different approach than the delivery of small molecules due to fast degradation in the gastrointestinal tract and rapid clearance from the body. Therefore, over the last decades, controlled drug delivery systems have been intensively investigated for the delivery of therapeutic proteins via alternative routes. For these drug delivery systems, the application of biodegradable polymers as carriers is considered one of the most promising options in this field.[1–4] The enormous variety of polymer systems available allows the development of customized formulations. Nevertheless, there are still major challenges to overcome before controlled delivery of proteins can become an established option in therapy. One of the major obstacles is maintaining the stability of proteins during production, storage and release.[5]

For long-term delivery, proteins can be encapsulated in biodegradable polymers in various injectable formulations, with microparticles and gels being most frequently investigated.[6–11] During the production of these formulations, the proteins are exposed to harsh conditions, *e.g.* shear, liquid-liquid, liquid-solid and liquid-air interfaces. These harsh conditions can induce protein deterioration, resulting in decreased biological activity and even in unwanted immune responses.[5,12,13]

A simple and extensively studied method to produce polymeric formulations containing small molecules or peptides is hot melt extrusion (HME).[14] During HME, a mixture of the polymer and drug in the dry state are fed into a funnel, which leads to the conveying system. The conveying system mixes and heats the constituent while transporting them to a die. The die molds the material into the desired shape, usually a rod or a thin film. Unlike other production techniques, such as emulsification as used for microparticle production, HME does not require the use of solvents. Consequently, this approach eliminates a major source of protein destabilization, as no water-organic solvent interfaces are created. Furthermore, HME is a fast, reproducible and continuous process and relatively easy to scale up.[15,16] However, the disadvantage of HME is that the drug is exposed to heat and shear, which may cause stability issues, especially for heat labile drugs as proteins.

In most polymer-based parenteral controlled and sustained release formulations, including implants produced by HME, poly(lactic-co-glycolic acid) (PLGA) is the most widely used polymer.[7] This polymer has various favorable properties, like its

biocompatibility and biodegradability, and moreover, it has been approved by the FDA for use in humans. However, the compatibility of PLGA with proteins is limited, as liquefying this polymer during HME temperatures of 85 °C or higher are required, which may cause stability issues with thermolabile compounds like proteins. Moreover, the hydrophobic surface of the polymer and its acidic degradation products may lead to detrimental interactions with proteins.[17,18]

The detrimental effects of PLGA on proteins have fueled the research for developing alternative polymeric systems with more favorable properties. Phase separated multi-block copolymers composed of poly(ε-caprolactone) (PCL) and poly(ethylene glycol) (PEG) may be an interesting alternative.[10] These polymers have a phase separated morphology of hydrophilic amorphous PEG-containing domains, *i.e.* [PCL-PEG-PCL] blocks, and hydrophobic crystalline PCL domains, which act as physical crosslinks. The incorporation of PEG in these polymers makes them more hydrophilic than PLGA and similar polymers. It has been hypothesized that these hydrophilic domains can shield the protein from hydrophobic surfaces within the formulation, thereby preventing physical or chemical degradation of the protein.[10] Furthermore, these phase separated multi-block copolymers enable sustained release of proteins by diffusion through the swollen polymer network,[19] which in addition may prevent the accumulation of acidic degradation products.[10,20] Another favorable property for protein formulation is that HME of [PCL]-*b*-[PCL-PEG-PCL] multi-block copolymers can be performed at a relatively low extrusion temperature of 55 °C. Stankovic *et al.* showed that the biological activity of lysozyme was fully preserved after HME in this polymer.[10,19]

The crystalline block in the abovementioned multi-block copolymers can be varied, for example by using poly (L-lactic acid) (PLLA) or blocks composed of an undisclosed alternative polyester (PEX), providing alternative release- and degradation kinetics. However, although having advantageous hydrophilic properties, these multi-block copolymers require higher extrusion temperatures, which are comparable with PLGA. To our knowledge, the effects of HME temperature and hydrophilicity of the polymers on the stability of proteins have not been systematically investigated yet.

Because we envisage that during HME at high temperatures using hydrophobic polymers proteins will be prone to degradation, pre-stabilization of the proteins might be beneficial. One approach to achieve this, is to incorporate them in a sugar glass matrix. For example, trehalose and inulin glasses have been shown to be

excellent protein stabilizers.[21,22] A recent study showed that the use of trehalose in hot melt extruded PLGA implants containing bovine serum albumin (BSA) prevents aggregation of the protein to a large extent.[23] However, the knowledge of the effect of sugar glasses on protein activity is limited to data of BSA, which is considered to be very stable.

Taking these recent developments together, we hypothesize that the application of the abovementioned hydrophilic multi-block copolymers extruded at low temperatures in combination with sugar glass pre-stabilization of the protein will minimize the degradation of heat labile proteins during HME. Therefore, in this study, we aim to investigate the influence of the above mentioned factors on the stability of two thermolabile model proteins, β-galactosidase[24] and alkaline phosphatase,[25] during the hot melt extrusion process. The enzymatic activity of the proteins was used as a measure for their stability.

MATERIALS AND METHODS

Materials

β-Galactosidase (βG) was purchased from Sorachim (Lausanne, Switzerland). Alkaline phosphatase (AP), ammediol, BSA, copper sulfate pentahydrate, disodium hydrogen phosphate dodecahydrate, magnesium chloride hexahydrate, *ortho*-nitrophenyl-β-galactoside, *para*-nitrophenylphosphate, poly-ε-caprolactone (45 kDa), sodium carbonate, sodium deoxycholate, monosodium phosphate dihydrate, sodium hydroxide, sodium tartrate and trichloroacetic acid were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Inulin (4 kDa) was kindly provided by Sensus (Roosendaal, The Netherlands). PLGA Low Molecular weight (PLGA LMw) and High Molecular weight (PLGA HMw) (PLDG 5002 and PLDG 5010, respectively) were obtained from Corbion (Gorinchem, The Netherlands). Folin-Ciocalteu phenol reagent was obtained from Merck Millipore (Amsterdam-Zuidoost, The Netherlands). Ethyl acetate was purchased from VWR (Amsterdam, The Netherlands). Pierce™ 660 nm Protein Assay Reagent was obtained from Life Technologies (Bleiswijk, The Netherlands). Sodium dodecyl sulfate was purchased from Spruyt-Hillen (IJsselstein, The Netherlands).

The phosphate buffers used in this study (0.1 M and 0.01 M) consisted of disodium hydrogen phosphate dodecahydrate and monosodium phosphate dihydrate in a 3:1 ratio; pH was adjusted to 7.3 using 1 M sodium hydroxide.

Methods

Polymer synthesis and characterization

The PCL-based hydrophilic multi-block copolymer, composed of 50 wt.% PCL blocks with a molecular weight of 4000 g/mole and 50 wt. % [PCL – PEG – PCL] blocks containing PEG1500 and with an overall molecular weight of 2000 g/mole, were synthesized and characterized as described by Stankovic *et al.*[10,19]. The PEX- and PLLA-based hydrophilic multi-block copolymers were synthesized and characterized by similar methods using PEX blocks with a molecular weight of 2500 g/mole and PLLA blocks with a molecular weight of 4000 g/mole as semi-crystalline blocks. The [PCL-PEG-PCL] block had an overall molecular weight of 2000 g/mole, containing PEG1500 in [PCL-PEG-PCL]-*b*-PEX and PEG1000 in [PCL-PEG-PCL]-*b*-PLLA. **Figure 4.1** summarizes the composition of the hydrophilic multi-block copolymers. Characteristics of all polymers used are listed in **Table 4.1**.

TABLE 4.1: Characteristics of polymers.

Polymer	IV (dl/g)	T_g (°C)	T_m (°C)	Extrusion temperature (°C)	Contact angle
PCL	N.A.	N.A.	58	55	70°
PCL-based	0.8	-58	50		21°
PLGA LMw	0.2	36	N.A.	85	72°
PEX-based	0.9	-60	77		59°
PLGA HMw	1.0	46	N.A.	130	70°
PLLA-based	0.8	-57	130		53°

In addition, the water contact angle on the polymer surface was determined. Polymers were dissolved in 1,4-dioxane in a 10 to 25% concentration and spin coated on a glass surface. Contact angles were measured at room temperature with an in-house developed contour monitor using the static sessile drop technique. Water drops of 1-1.5 mL were positioned on the surface and the contact angle was monitored for 20 to 40 s. The value after stabilization was taken as the contact angle. The contact angle

measurements confirmed the hydrophobic nature of PCL and both PLGAs **(Table 4.1)**, while the PEG-containing PCL-based, PEX-based and PLLA-based multi-block copolymers, having lower contact angles, are considered as hydrophilic polymers.

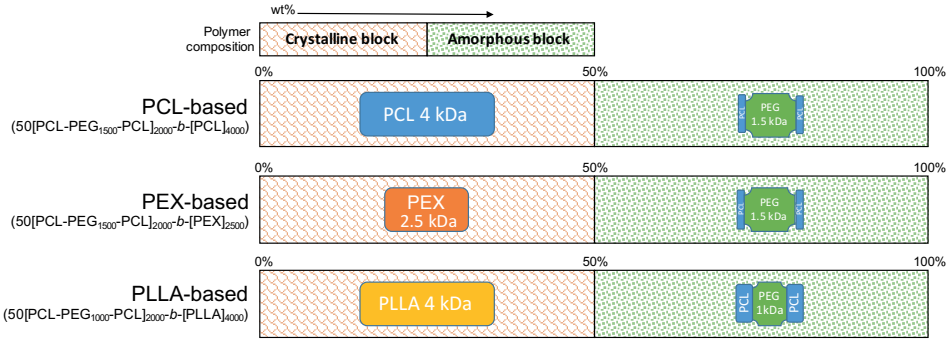

FIGURE 4.1: Graphical representation of the composition of synthesized phase-separated multi-block copolymers.

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Spray drying

Spray drying was performed using a Büchi B-290 mini spray drier (Büchi, Flawil, Switzerland) equipped with a standard two fluid nozzle. AP and βG (2.5 mg/mL) were dissolved in demineralized water and 0.01 M phosphate buffer, respectively, and mixed with inulin solution (25 mg/mL) in the same solvent to obtain a 1:10 protein:inulin weight ratio. Spray drying was performed with a feeding rate of 2.5 mL/min and an inlet temperature of 100°C (measured outlet temperature was 64-66 °C). The atomizing airflow was set at 500 L_n/h and the aspirator at 100%.

Physico-chemical characterization of spray dried powders

The water content of AP spray dried with inulin (AP-IN) and βG spray dried with inulin (βG-IN) was determined (in duplicate) by Karl Fisher coulometric water titration using an 831 KF Coulometer (Metrohm Applikon, Schiedam, The Netherlands).

The glass transition temperatures (T_g) of AP-IN and βG-IN were determined using Differential Scanning Calorimetry (DSC). An amount of 2 to 4 mg of sample was accurately weighed in open aluminum pans and analyzed using a Q2000 DSC (TA Instruments, Ghent, Belgium). Samples were preheated for 3 min at 80 °C to remove

residual water. Next, the temperature was equilibrated at 20 °C and subsequently raised to 180 °C at a rate of 20 °C/min. The inflection point of the heat flow versus temperature curve was taken as T_g.

In addition, the effect of the residual water on the T_g was analyzed using Modulated DSC (MDSC). An amount of 2 to 4 mg of sample was accurately weighed in aluminum pans, which were hermetically sealed subsequently, and analyzed using a Q2000 DSC. The temperature was equilibrated at 0 °C for 5 min. Next, the temperature was raised at a rate of 1 °C/min to 120 °C with a modulation of ±0.159 °C every 60 s. The inflection point of the reversing heat flow versus temperature curve was taken as T_g.

Heat stress on dry powders

To assess the effect of heat on dry powders, proteins as received and protein/inulin spray dried powders were exposed to 55, 95 and 130 °C for 10, 30, 60 and 120 min (n=3). To analyze the temperature dependency of the degradation process more accurately, additional samples (n=3) were exposed to 37 °C for a maximum of 4 weeks and to 55 °C for a maximum of 10 days. An amount of 1-3 mg powder was accurately weighed in 4 mL glass vials and placed uncapped in a preheated oven. After removal, the vials were capped immediately and cooled to room temperature. The powders were then reconstituted in demineralized water (AP) or 0.1 M phosphate buffer (βG) and analyzed for enzyme activity.

Hot melt extrusion

The production of implants by HME was performed using a HAAKE MiniLab Rheomex CTW5 co-rotating twin-screw extruder (Thermo-electron). A 5 g powder blend with a final protein content of 1% was prepared by manual mixing of the polymer and protein as received or protein/inulin spray dried powder. Extrusion was performed with a screw speed of 2-15 rpm, developing a torque of 4-10 N/cm. The extrusion temperature depended on the polymer **(Table 4.1**). The extruded samples were stored at -20 °C until further analysis.

Extraction of protein from extrudates

Random samples of extrudate were taken at the start, middle and end of the process (n=6). Approximately 10 mg of extrudate was accurately weighed and 1 mL of ethyl acetate was added. After the polymer was completely dissolved, the samples were centrifuged for 15 min at 13,000 rpm (Microcentrifuge SIGMA 1-14) and

the supernatant was removed. The washing steps were repeated three times. The remaining pellet was dried overnight to remove residual solvent. The samples were then reconstituted in demineralized water (AP) or 0.1 M phosphate buffer (βG) and analyzed for protein content and activity. The extraction process was shown not to affect the enzymatic activity of the proteins (data not shown).

Protein content of extrudates

The protein content of AP samples was determined after extraction with a modified Lowry assay as described by Tonnis *et al.*.[26] Briefly, protein was precipitated in an aqueous solution containing 0.025% (w/v) sodium deoxycholate and 6% (w/v) trichloroacetic acid. After 30 min incubation on ice, the samples were centrifuged for 15 min at 13,000 rpm (Microcentrifuge SIGMA 1-14) and the supernatant was removed. Next, the pellet was reconstituted in Lowry reagent (2% sodium carbonate, 0.5% sodium tartrate and 2.5% sodium dodecyl sulfate in 0.1 M NaOH) and analyzed with the Lowry assay using Folin-Ciocalteu phenol reagent. Protein concentrations were calculated from a 6-point calibration curve.

The protein content of βG samples was determined with the Pierce 660 nm assay (Thermo Fisher Scientific, Etten-Leur, The Netherlands). After reconstitution in 1.0 mL 0.1 M phosphate buffer, 50 µL of sample was transferred to a 96 wells plate and 150 µL of Pierce 660 nm reagent was added to each well. After incubating for 5 min, the absorbance was measured at 660 nm (Synergy HT Microplate Reader, BioTek Instruments, Winooski, VT). Protein concentrations were calculated from a 7-point calibration curve.

Enzymatic activity of proteins

The activity of AP was determined using a kinetic enzymatic assay based on a method described by Grasmeijer *et al.*.[27] In brief, 20 µL of sample (7.5 µg/mL) was pipetted into each well of a BSA-coated 96-well plate (Greiner, F-shape). After the addition of 190 µL of 1.2 µM $MgCl_2$ in 50 mM ammediol pH 9.8 buffer to each well, the plate was incubated at 37°C for 10 min. Finally, 20 µL of 42.5 mg/mL *para*-nitrophenylphosphate was added and the conversion to *para*-nitrophenol was monitored at 37 °C by measuring the absorbance at 405 nm every 30 s for 15 min (Synergy HT Plate Reader). The slope of the conversion was used to calculate the activity in units/mg.

The activity of βG was determined with a kinetic enzymatic assay based on a method described by Tonnis *et al.*.[22] In brief, samples (5 μg/mL) were prepared in 0.1% BSA, 1 mM $MgCl_2$, 50 mM phosphate buffer. 20 μL sample and 200 μL $MgCl_2$ solution (1.4 mM $MgCl_2$ in 0.1 M phosphate buffer, pH 7.3) were pipetted into each well of a 96-well plate (Greiner, F-shape) and the plate was incubated at 37 °C for 10 min. Next, 20 μL of 50 mM *ortho*-nitrophenyl-β-galactoside was added to the wells. The conversion to *ortho*-nitrophenol was determined by measuring the absorbance at 405 nm every 30 s for 15 min at 37°C (Synergy HT Plate Reader) and the activity in units/mg was calculated from the slope of this conversion.

Data analysis

Two-way ANOVA followed by Sidak multiple comparisions test and non-linear regression analysis were performed using GraphPad Prism, version 6.00 (GraphPad software, La Jolla, CA). Error bars in all graphs indicate standard deviation.

RESULTS

Physical-chemical properties of spray dried powders

The enzymatic activity of both AP and βG was fully preserved after spray drying **(Table 4.2)**. No major differences between AP-IN and βG-IN with respect to T_g values of the anhydrous and hydrated powders, and the water content were observed **(Table 4.2)**.

TABLE 4.2: Glass transition temperatures (T_g) and water content of AP-IN and βG-IN.

	Activity after spray drying % (SD)	T_g anhydrous powder (°C)	T_g including residual water (°C)	Residual water content (%)
AP-IN	98.2 (5.0)	157.8	63.8	8.3
βG-IN	106.4 (13.8)	169.5	65.8	7.9

Heat stress on Alkaline Phosphatase

The conditions for the heat stress experiments were chosen to mimic HME conditions used in this study in terms of temperature and exposure time. Exposure at 55 °C for 120 min did not lead to a change in activity for either bare AP or AP-IN (**Fig. 4.2A**).

The same result was observed for AP-IN when exposed at 95 °C for 120 min, whereas AP showed a decrease in activity of about 30% at this temperature ($p \leq 0.0001$) (**Fig. 4.2B**). Furthermore, exposure to 130 °C for 120 min caused considerable activity loss, which resulted in residual activities of 16% for AP and 65% for AP-IN (**Fig. 4.2C**). These results indicate that incorporation in inulin can reduce the degradation rate of AP when dry heat stress is applied to solid powders. However, at 130 °C, inulin inclusion could strongly diminish the activity loss of AP, but not fully prevent it.

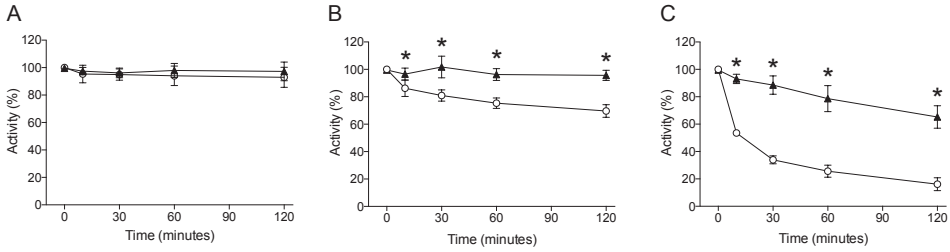

FIGURE 4.2. Residual activity of bare alkaline phosphatase (○)and spray dried alkaline phosphatase with inulin (▲) after exposure to dry heat of 55 °C (A), 95 °C (B) and 130 °C (C). *$p<0.05$ between formulations.

Heat stress on β-Galactosidase

After exposure to 55 °C and 95 °C, bare βG and βG-IN showed no difference in activity loss (**Fig. 4.3**). As with AP and AP-IN, exposure to 55 °C caused no activity loss. Conversely, exposure to 95 °C for 120 min resulted in an activity loss of 14% and 18% for βG and βG-IN, respectively ($p \leq 0.0001$). Remarkably, βG samples performed better when exposed to 130 °C than βG-IN samples did: the residual activities were 44% for βG and 7% for βG-IN after 120 min. These results suggest that inulin did not protect βG from degradation, but even had a detrimental effect on stability when exposed to these extreme temperatures.

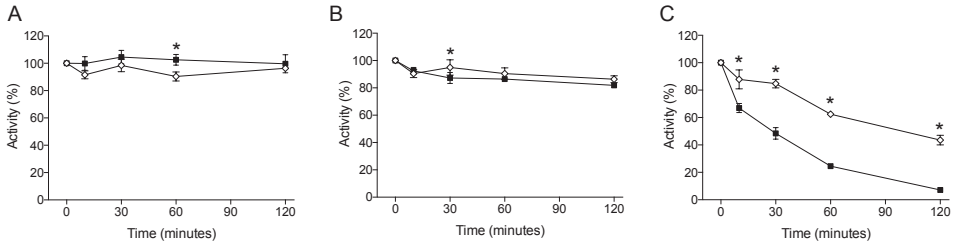

FIGURE 4.3: Residual activity of bare β-galactosidase (◇) and spray dried β-galactosidase with inulin (■) after exposure to dry heat of 55 °C (A), 95 °C (B) and 130 °C (C). *p<0.05 between formulations.

Temperature dependence of activity loss

The temperature dependency of the degradation after heat stress was assessed by analyzing the relationship of the rate constant of the reaction and the temperature in an Arrhenius plot (**Table 4.3** and **Fig. 4.4**). The correlation coefficients indicate that all the powders followed Arrhenius kinetics for activity loss during heat stress. The energy of activation, E_a, was calculated from the slope of the curve, which can be described by

$$\ln k = \ln A - \frac{E_a}{R}\frac{1}{T} \tag{4.1}$$

in which k is the reaction rate constant, A is the Arrhenius factor, R is the gas constant and T is the absolute temperature. Similar values of E_a were found for AP-IN and βG-IN (86.1 kJ K^{-1} mol^{-1} and 89.3 kJ K^{-1} mol^{-1}, respectively), whereas for AP a higher (107.5 kJ K^{-1} mol^{-1}) and for βG a lower value was calculated (77.5 kJ K^{-1} mol^{-1}). The relative temperature dependency of the degradation rate during heat stress can be described as βG < AP-IN ≈ βG-IN < AP.

Hot melt extrusion

The run time for extrusion of 5 g of polymer/protein mixtures was between 40 and 60 min. Samples were collected throughout the extrusion run and 8 or more samples were assessed for protein content and enzymatic activity after HME. We aimed for extrusion at the same temperatures as used in the heat stress experiments. However,

for the intermediate temperature of 95 °C, the polymer was liquified to a degree that the viscosity was too low to create rod-shaped solid implants. Therefore, we used a slightly lower extrusion temperature of 85 – 90 °C for these samples.

TABLE 4.3: First order degradation rate constants of bare and spray dried proteins.

	First order degradation rate constant *k* (h^{-1})			
	37 °C	**55 °C**	**95 °C**	**130 °C**
AP	$-2.219*10^{-4}$	$-6.937*10^{-4}$	-0.179	-1.854
AP-IN	$-1.155*10^{-4}$	$-4.290*10^{-4}$	-0.019	-0.217
βG	$-6.392*10^{-4}$	$-1.096*10^{-3}$	-0.059	-0.424
βG-IN	$-5.835*10^{-4}$	$-1.994*10^{-3}$	-0.090	-1.485

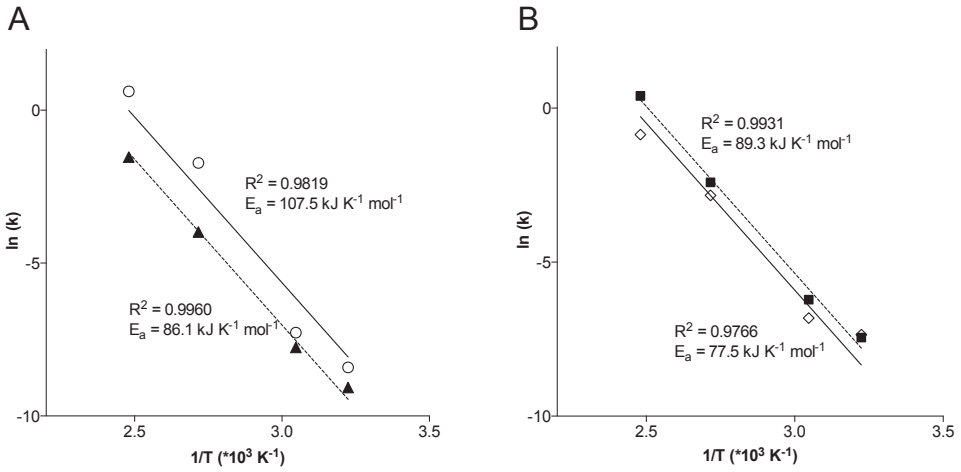

FIGURE 4.4: Arrhenius plots of activity loss after exposure to dry heat of (A) bare alkaline phosphatase (○, solid line) and spray dried alkaline phosphatase with inulin (▲, dashed line) and (B) bare β-galactosidase (◇, solid line) and spray dried β-galactosidase with inulin (■, dashed line).

Alkaline Phosphatase

The effect of the extrusion temperature on the activity of (pre-stabilized) AP is depicted in **Figure 4.5**. The overall trend is that the activity after HME of AP and AP-IN decreased with increasing temperature. The threshold temperature for the onset of

this decrease seems to be higher for AP-IN than for AP; a major reduction in activity was already apparent at 85 °C in the AP samples, while the AP-IN samples showed a smaller (PEX-based) or no (PLGA LMw) reduction in activity at this temperature.

Pre-stabilization of AP with inulin showed no beneficial effect on stability after HME at 55 °C or 130 °C. At a low extrusion temperature of 55 °C, all formulations showed high remaining activity of >75%. However, at a high extrusion temperature of 130 °C, the remaining activity was <8% for all formulations. At the intermediate temperature of 85 °C, the stabilizing effect of inulin during HME is evident, as the remaining enzymatic activities of AP-IN were 2.3 and 3.1 times higher for PLDG 5002 and the PEX-based polymer, respectively, than when the bare protein was used. The use of the hydrophilic multi-block copolymers instead of the hydrophobic polymers showed no beneficial effect on the activity of AP after HME.

These results indicate that the activity loss of AP during HME is most strongly affected by the extrusion temperature. However, at intermediate temperatures, pre-stabilizing with inulin can inhibit this effect.

β-Galactosidase

In contrast to AP, βG and βG-IN did not show a decrease in activity with increasing extrusion temperature **(Figure 4.6)**. Remarkably, the highest activity was found after extrusion of the proteins with the PEX-based polymer at 85 °C.

Pre-stabilization of βG with inulin had a positive effect on the activity after HME in more conditions than for AP, but the effect was less pronounced. Extrusion at 55 °C and 85 °C resulted in a higher recovery of enzymatic activity using βG-IN – the activity increased between 1.2 to 1.6-fold – than when the bare protein was used. In contrast, the protein was almost fully degraded when βG-IN was used during extrusion at 130 °C, whereas it remained relatively stable when using bare βG.

The incorporation of PEG in the polymer resulted in increased stability during HME in most but not all cases. Clearly, the use of the PEX-based polymer was favorable compared to PLGA LMw (extruded at 85 °C) for βG stability for both the bare and pre-stabilized protein. Likewise, a higher stability of the protein in a PEG-containing polymer was observed for βG-IN at 55 °C and βG at 130 °C. However, the stability

was not significantly different for βG at 55 °C and βG-IN at 130 °C. Evidently, there was no trend of the effects of more hydrophilic polymers, or the incorporation of PEG related to the application of pre-stabilization or the extrusion temperature.

In summary, the activity of βG after HME is only to a limited extent related to extrusion temperature. Pre-stabilization with inulin slightly increased stability of βG when extruded at 55 °C and 85°C but had a detrimental effect when extruded at 130 °C. The use of hydrophilic polymers was beneficial for stability in some but not all conditions.

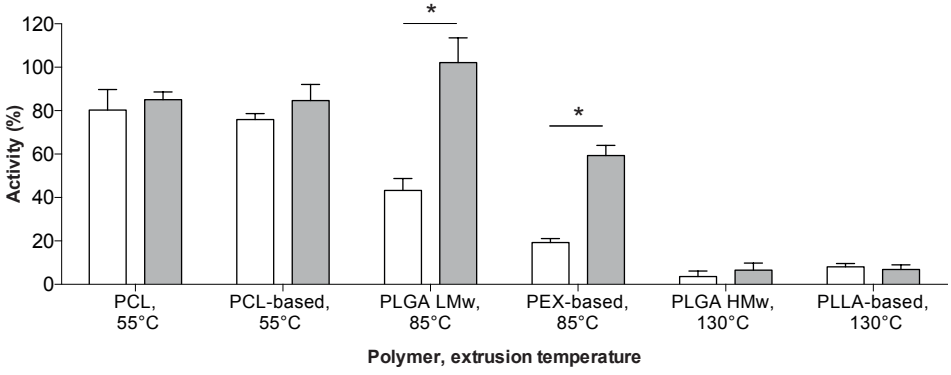

FIGURE 4.5: Protein activity of bare alkaline phosphatase (white bars) and spray dried alkaline phosphatase with inulin 1:10 w/w (grey bars) after hot melt extrusion in different polymers. *p<0.0001.

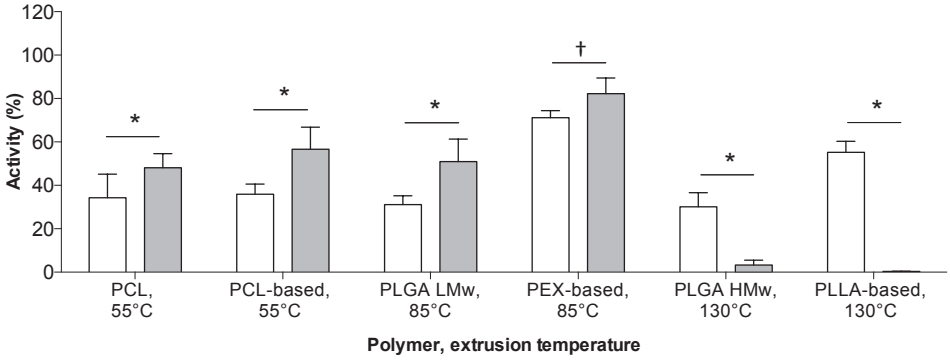

FIGURE 4.6: Protein activity of bare β-galactosidase (white bars) and spray dried β-galactosidase with inulin 1:10 w/w (grey bars) after hot melt extrusion in different polymers. †p<0.001, *p<0.0001.

DISCUSSION

In this study, we hypothesized that a low extrusion temperature, sugar glass pre-stabilization of proteins and the use of polymers with hydrophilic regions could reduce protein degradation during HME. We found that using a low extrusion temperature and using sugar glass pre-stabilization at intermediate extrusion temperature improved protein stability. In contrast, the use of hydrophilic multi-block copolymers had a variable effect on the stability of proteins during HME.

As opposed to dry heat exposure at low temperature, at intermediate and high temperatures, a stabilizing excipient is necessary for preservation of the enzymatic activity for both proteins. However, at these temperatures, major differences in preservation of enzymatic activity were observed between the proteins studied. The stabilizing effect of inulin incorporation on AP is in accordance with a study of Ragjagopal *et al.*,[23] in which it was shown that upon exposure to 100 °C, incorporation of BSA in trehalose prevented aggregation of this protein compared to non-stabilized BSA. Conversely, inulin incorporation showed no stabilizing effects on βG at high temperatures. The samples were exposed to dry heat in open vials and most if not all residual water is expected to rapidly evaporate, raising their T_gs to above storage temperature. Despite the fact that the samples were in the glassy state during dry heat exposure, it has been reported that substantial molecular mobility still occurs at temperatures as low as 50 °C below T_g.[28,29] However, Grasmeijer *et al.*[27] showed that maximal storage stability of AP incorporated in inulin is maintained at temperatures up to 15 °C below the T_g. This is in accordance with our findings on the dry heat exposure of AP, but it does not seem to apply to βG. Possibly, the threshold temperature for maximal stabilization is protein dependent.

The calculated values for the activation energy E_a (Eq. 4.1) of degradation during dry heat exposure correlated in general well with the relative temperature dependency of the stability of the proteins during HME. Bare AP, having the highest E_a value, also shows the largest decrease in activity with increasing extrusion temperatures. Conversely, the remaining activity of bare βG was affected less by temperature, which is reflected in the lowest E_a value.

Furthermore, the dry heat exposure results were predictive for the effects of inulin during HME, as similar trends are observed for all four protein formulations. For example, after extrusion at 85 °C, the activity of AP-IN was significantly higher than

that of AP, which was also observed in the dry heat exposure experiments. Inulin has been thoroughly investigated as a stabilizer for biopharmaceuticals during drying and subsequent storage.[22,27,30] Furthermore, inulin has been used before as an initial processing step for proteins[19] and as a release controlling excipient in polymer-based implants produced by HME,[10] but its protective effect during HME had not been investigated yet. Our results show that inulin incorporation can improve the stability of proteins during extrusion at relatively low and intermediate temperatures. However, this effect is not significant if the bare protein itself is only slightly affected by the process, as is demonstrated with extrusion of AP at 55 °C. Conversely, at an extrusion temperature of 130 °C the use of inulin does not improve stability, as in the case of AP, and can even be detrimental, as found for βG. In contrast to the dry heat exposure experiments, evaporation of residual water is probably limited during HME. Residual water lowers the T_g, which will thus be in between the anhydrous T_g and the T_g after spray drying **(Table 4.2)** and most likely below the extrusion temperature of 130 °C. Thus, by being in the rubbery state, inulin incorporation may fail to stabilize the protein and it may even act as a destabilizer. This was supported by the observation that during extrusion of the pre-stabilized proteins at this high temperature, the extrudate showed increasing browning. This is indicative of caramelization of inulin or the Maillard reaction, as commercial batches of inulin usually contain some reducing groups.[22,30] Moreover, activity loss of proteins can be accelerated by degrading sugars when exposed to compression forces,[31] like during HME.

The remaining protein activity after HME was considerably lower than after dry heat exposure only, particularly in the case of βG. As mentioned above, a lowered T_g due to residual water can impair the stability, also in bare proteins. Furthermore, heat is not the sole stress factor during HME. In particular, shear and compression forces may cause major stresses on the constituents during the extrusion process. Therefore, the difference between the remaining activity after dry heat exposure and after HME could partially be attributed to these stresses. Lastly, having a very high molecular weight of 540 kDa, βG might be more susceptible to shear than the smaller AP, which has a molecular weight of 160 kDa.

The incorporation of PEG into polymers was hypothesized to be beneficial for protein stability during extrusion due to its hydrophilic and thus more protein compatible nature. The effect would be that hydrophobic interactions are decreased.[10] Indeed, polymers containing PEG showed lower contact angles with water than the

hydrophobic polymers, which indicates a more hydrophilic nature. In this study, the use of hydrophilic polymers did in some cases indeed lead to improved protein stability. However, in other cases no or even negative effects were seen. Likewise, Lee *et al.*[32] showed that the addition of PEG 6000 to PLGA did not result in improved protein activity after melt extrusion. Apparently, hydrophilic regions, either incorporated in the polymer or manually mixed with the polymer, have unpredictable effects on the stability of proteins during HME. Although the swelling of the PEG domains of the hydrophilic multi-block copolymers allows diffusion controlled release of proteins,[19] it remains to be investigated whether the hydrophilic nature by itself could be advantageous during storage and release.

HME of mixtures of proteins and polymers to obtain controlled release systems is a relatively new approach, for the most part because the harsh conditions of this process were thought to be unsuitable for fragile products like proteins. Stankovic *et al.*[19] already showed that several proteins remain intact after extrusion at 55 °C in similar multiblock copolymers containing hydrophilic PEG blocks as used in this study. In continuation of this work, the present study explores the possibilities and limitations of HME for protein incorporation by selecting fragile proteins and extreme processing temperatures. Our experiments confirmed that extrusion of these fragile proteins in polymers with preservation of the activity is possible at extrusion temperatures up to at least 85 °C and in different types of polymers. Moreover, pre-stabilization of proteins by inulin incorporation can also improve the stability during HME. Furthermore, thermal stability data of the bare protein or the sugar incorporated protein can be applied to assess the possible temperature range for extrusion and thereby provide guidance in the selection of the polymer. These findings open wider possibilities for the development of controlled release polymeric formulations containing therapeutic proteins using HME. Combined with the multiple advantages of this production technique over conventional batch processes for protein encapsulation, HME can now be regarded as a potent production process for the manufacturing of sustained release formulations for therapeutic proteins.

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Abbreviations

HME, hot melt extrusion; βG, β-galactosidase; AP, alkaline phosphatase; βG-IN, β-galactosidase spray dried with inulin; AP-IN, alkaline phosphatase spray dried with inulin; PLGA, poly (lactic-co-glycolic acid); PCL, poly (ε-caprolactone); PEG, poly (ethylene glycol); PEX, undisclosed Polyester X; PLLA, poly (L-lactic acid); PLGA LMw, low molecular weight poly (lactic-co-glycolic acid); PLGA HMw, high molecular weight poly (lactic-co-glycolic acid); BSA, bovine serum albumin; T_g*, glass transition temperature;* T_m*, melting temperature; IV, intrinsic viscosity;* E_a*, energy of activation; R, gas constant; T, absolute temperature; A, Arrhenius factor; DSC, differential scanning calorimetry; MDSC, modulated differential scanning calorimetry.*

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